IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) :	Wu et al.	)	Examiner:
		)	Michail A. Belyavskyi
Serial No. :	10/679,184	)	
		)	Art Unit:
Cnfrm. No. :	2775	)	1644
		)	
Filed :	October 3, 2003	)	
		)	
For :	THREE-DIMENSIONAL PERIPHERAL LYMPHOID ORGAN CELL CULTURES	)	
		)	

DECLARATION OF ANDREA BOTTARO UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, ANDREA BOTTARO, pursuant to 37 C.F.R. § 1.132 declare:

1. I received a first-level doctorate ("Laurea") in Biology from the University of Torino, Italy, in 1987, and a Research Doctorate (Ph.D. equivalent) in Human Genetics from the University of Torino, Italy, in 1993.

2. I am currently an Associate Professor of Medicine, Microbiology and Immunology, and Oncology, at the School of Medicine and Dentistry, University of Rochester, Rochester, New York.

3. I am a co-inventor of the above-identified patent application.

4. I understand that the U.S. Patent and Trademark Office considers that WO 01/036589 to Wu et al. ("Wu"), WO 99/15629 to Pykett et al. ("Pykett"), and U.S. Patent No. 5,160,490 to Naughton et al. ("Naughton") each teach a method for culturing peripheral lymphoid organ cells by culturing the cells on a three-dimensional scaffolding, which is covered

or surrounded with culture medium, under conditions effective to generate and maintain mature and functional peripheral lymphoid organ cells, where the three-dimensional scaffolding allows cells in the culture medium to have cell to cell contact in three dimensions. I have reviewed Wu, Pykett, and Naughton and am presenting this declaration to demonstrate that these references do not teach a method for culturing peripheral lymphoid organ cells under conditions effective to generate and maintain mature and functional peripheral lymphoid organ cells.

5. Wu teaches a cell culture system that includes a three-dimensional support for the culture of hematopoietic stem cells and stromal cells, and media that supports the growth or differentiation of the stem cells into immune system cells. Wu specifically describes culturing the mononuclear cell layer of human bone marrow in a three-dimensional bioreactor, and reports that the culture resembled the function of bone marrow *in vivo*. *See* Wu at 32–40. Pykett has been cited for teaching a method of culturing, on a three-dimensional porous biomaterial, hematopoietic progenitor cells in the absence of exogenously added hematopoietic growth factors. Pykett specifically describes carrying out its method using hematopoietic progenitor cells derived from human bone marrow. *See, e.g.*, Pykett at 26–27. Naughton teaches a method for culturing cells and tissues *in vitro* for prolonged periods of time. This method involves culturing cells derived from a desired tissue on a pre-established stromal support matrix. Naughton specifically describes carrying out its method with bone marrow cells, skin cells, liver cells, mucosal epithelial cells, pancreatic cells, brain cells, and adenocarcinoma cells. *See, e.g.*, Naughton at §§ 11–17. I understand that the PTO considers the bone marrow-derived hematopoietic stem cells of Wu, Pykett, and Naughton to be synonymous with peripheral lymphoid organ cells of our present invention. I disagree.

6. Bone marrow is the primary hematopoietic organ in mammals. Hematopoietic cells in bone marrow are phenotypically and functionally distinct from hematopoietic cells in the periphery (*i.e.*, in blood, lymph, peripheral lymphoid organs, and other tissues). These differences are especially crucial in the case of hematopoietic cells involved in immune responses, because these cells *must* complete their maturation in the periphery to become fully functional, as highlighted below.

7. Briefly, the bone marrow is the site where hematopoietic cells (lymphocytes; myelocytes, *i.e.*, granulocytes, monocyte/macrophages, platelets and certain kinds of dendritic cells; and red blood cells) differentiate from common precursor stem cells. This

initial stage of differentiation leads to generation of immature cells that leave the bone marrow and enter the periphery. In the case of lymphocytes (B and T cells), the principal effectors of adaptive immune responses in mammals, the maturation is only completed after exiting the bone marrow. T cell precursors from the bone marrow need to undergo a complex maturation process in the thymus, where they are selected for their ability to discriminate histocompatibility antigens on the surface of other cells. Hogquist et al., "Central Tolerance: Learning Self-control in the Thymus," *Nat. Rev. Immunol.* 5:772–82 (2005) (Exhibit 1). B cells must also complete their maturation in the periphery, also becoming competent in their ability to distinguish foreign substances from "self." These maturation steps involve significant changes in the phenotype of cells (expression of specific molecules on the cell surface) as well as in their functional properties (ability to respond to stimuli, "homing" to specific tissues and sites, etc.). Cancro, "Peripheral B-cell Maturation: The Intersection of Selection and Homeostasis," *Immunol. Rev.* 197:89–101 (2004) (Exhibit 2). For instance, B lymphocytes newly generated from the bone marrow respond to stimulation of their surface antibody molecules by either dying (apoptosis) or by becoming inactivated, but the same stimulus delivered to a mature B cell triggers cell activation and involvement in an immune response. Chung et al., "CD23 Defines Two Distinct Subsets of Immature B Cells Which Differ in Their Responses to T Cell Help Signals," *Int'l Immunol.* 14(2):157–66 (2002) (Exhibit 3). Disruption of these peripheral maturation processes can lead to immunodeficiency or, at the other end of the spectrum, autoimmunity. Cornall et al., "The Regulation of Self-reactive B Cells," *Curr. Opin. Immunol.* 7:804–11 (1995) (Exhibit 4). Therefore, there are critical differences between the intrinsic features of hematopoietic cells, and especially immune system cells, residing in the bone marrow, and those residing in peripheral lymphoid organs. In short, hematopoietic stem cells derived from bone marrow are not peripheral lymphoid organ cells.

8. Moreover, I do not believe that a skilled scientist familiar with Wu, Pykett, and/or Naughton would have expected to be able to culture peripheral lymphoid organ cells. That is because salient differences exist in the ultra-structural and histological organization of peripheral lymphoid organs (*e.g.*, spleen, lymph nodes) and bone marrow, which reflect their different functions. The role of the bone marrow stroma is to support and sustain the maturation process of the hematopoietic precursors along various cell lineages, including lymphocytes. Derubeis & Cancedda, "Bone Marrow Stromal Cells (BMSCs) in Bone Engineering: Limitations

and Recent Advances," *Ann. Biomed. Eng.* 32(1):160–5 (2004) (Exhibit 5); Nagasawa, "Microenvironmental Niches in the Bone Marrow Required for B-cell Development," *Nat. Rev. Immunol.* 6:107–16 (2006) (Exhibit 6). Stromal cells in the peripheral lymphoid organs have the role of organizing the histology of the organ in specific zones as required for functional immune responses to develop. Mempel et al., "Rulers over Randomness: Stroma Cells Guide Lymphocyte Migration in Lymph Nodes," *Immunity* 25:867–9 (2006) ("Mempel") (Exhibit 7). In both the spleen and lymph nodes, specialized areas are identifiable where T cells and various subsets of B cells specifically reside (periarthericral lymphoid sheaths, B cell follicles, and marginal zones). Crivellato et al., "Setting the Stage: An Anatomist's View of the Immune System," *Trends Immunol.* 25(4):210–7 (2004) ("Crivellato") (Exhibit 8). These areas have no counterpart in the bone marrow, and correspond to the distribution of specific stromal cells (such as follicular dendritic cells) that develop *in situ* by effect of the mature lymphocytes themselves. This structural organization is key in generating and sustaining immune responses *in vivo*, in that the migration of antigen-specific lymphocytes in the appropriate areas where antigen may be presented to them by accessory cells, and their proliferation and differentiation, are tightly regulated processes whose disruption can lead to defective immune responses. Crivellato; Vinuesa & Cook, "The Molecular Basis of Lymphoid Architecture and B Cell Responses: Implications for Immunodeficiency and Immunopathology," *Curr. Mol. Med.* 1:689–725 (2001) ("Vinuesa") (Exhibit 9). Finally, immune responses culminate in the generation, within the structure of peripheral lymphoid organs, of unique histological sites called germinal centers, in which antigen-specific B cells, T cells, and follicular dendritic cells cooperate in the generation of both effector cells that mediate immune responses, as well as memory cells responsible for long-term immunity to pathogens (*e.g.*, following vaccine inoculation). McHeyzer-Williams et al., "Germinal Center Reaction," *Curr. Opin. Hematol.* 8:52–9 (2001) (Exhibit 10). Therefore, crucial differences exist between peripheral lymphoid organs and bone marrow in their histological, functional, and cellular properties.

9. For the reasons highlighted above, the behavior of hematopoietic lineage cells from peripheral lymphoid organs within a bioreactor microenvironment was neither directly expected nor predictable based on the methods taught by Wu, Pykett, and Naughton with respect to bone marrow hematopoietic cells. Peripheral lymphoid organ cells are exquisitely sensitive to specific microenvironmental signals delivered by both cell-cell interactions with cognate cells, as

well as by soluble factors permeating the various areas within the peripheral lymphoid organ, and disruption of these signals in culture conditions normally leads to rapid death or acquisition of unusual and potentially dysfunctional phenotypes. Crivellato; Mempel; Vinuesa. Therefore, I do not believe that the success in culturing bone marrow hematopoietic cells in a three-dimensional bioreactor described in Wu, Pykett, and/or Naughton would have lead a scientist to conclude that one could culture peripheral lymphoid organ cells under conditions effective to generate and maintain mature and functional peripheral lymphoid organ cells.

10. Our finding that bioreactor cultures are able to sustain for several weeks the presence of various subsets of peripheral lymphoid organ cells, which closely replicate, both phenotypically and functionally, those found in normal, intact peripheral lymphoid organs, was therefore both surprising and scientifically exciting.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

2/1/07
Date

Andrea Bottaro, Ph.D.